EXPERIMENTAL BONE MARROW TRANSPLANTATION

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Bone marrow transplantation has become feasible in recent years. There are three main indications for such a procedure: i) in cases of myeloid insuffiicency as replacement therapy; ii) in the treatment of certain cases of neoplasia; and iii) in the production of artificial chimeras so as to make possible tissue and organ transplantation (Mathe and Amiel, 1964).

Technically bone marrow transplantation is relatively easy ta perform, since it involves only the accumulation of large amounts of donor marrow, usually from multiple sites and from multiple donors — about 10^{10}—10^{11} cells are usually needed for a single transplant. The ability to store such cells at very low temperatures (about 150° C) with subsequent resumption of normal activity upon transplantation (Malinin *et al.*, 1964) has helped considerably to make this an attractive proposition.

Numerous attempts have been made to treat aplastic states with marrow transfusions. Isogenic grafts in the human are possible only in identical twins, and this presents no real problems. Patients given such isogenic bone marrow showed evidence of marrow regeneration within a few weeks after transplantation (Papiernik-Birkhauer *et al.*, 1963). The treatment of these states with allogeneic bone marrow, i.e. bone marrow from genetically different donors, (as is the case in the majority of human transplants) is complicated by phenomena of rejection and of Graft-versus-Host reactions. The former can occur in the patient with a normal immune system, whereas the latter occurs when the immune system has been suppressed to such an extent that the body cannot reject the injected cells. If they are themselves capable of immunological activity, the injected cells can actually attack the host cells immunologically, with the production of "secondary disease", characterized by: a) anorexia, nausea, vomiting, diarrhoea, emaciation, hepatomegaly, erythrodermia of desquamative kind; b) haematological changes, including lymphocytpoenia, and eosinophilia; c) histological changes, including aplasia of the intestinal crypts, hyperkeratotic skin lesions, lymphoid aplasia; and biochemical changes, such as changes in the gamma globulins and in various serum enzyme levels (Mathe and Amiel, 1964).

Bone marrow therapy for neoplastic conditions has been restricted mainly to cases of leukaemia (Haruani *et al.*, 1960, Mathe, 1960). Other workers have tried to use bone marrow actively to reject neoplastic cells. It has been shown that immunologically competent cells injected into a leukaemic subject can attack the leukaemic cells (Barnes and Loutit, 1957, Mathe and Bernard, 1959, Mathe *et al.*, 1962, De Vries and Vos, 1958), and solid tumour cells (Woodruff an dSymes, 1962). Experimental studies by Mathe *et al.* (1962) show that the marrow immunised against Charlotte Friend's viral leukaemia significantly prolonged the life of animals suffering from the disease. Preliminary results in man (Mathe and Amiel, 1964) show prolonged survival of leukaemic patients treated with bone marrow grafts.

The establishment of bone marrow grafts in irradiated or otherwise treated individuals can render the recipient a chimaera, with the possibility that subsesequent organ transplants (from the same donor) will have a better chance of survival. This phenomenon has been shown to obtain for skin or ovary transplants in mice (Armand and Smith, 1959), for kidney transplants in dogs (Mannick *et al.*,

1959), and for skin grafts in man (Mathe *et al.*, 1963, 64).

It can readily be appreciated, therefore why studies on bone marrow transplantation assume such fundamental importance. It is also obvious that studies that might shed any light on the problem of growth, division, and differentiation of bone marrow are likely to be of interest. With this in mind the following studies were undertaken.

Materials and methods

Mice of the CFW strain, weighing 20-25 gm. at the beginning of the experiment were used. Each group consisted of 5-10 mice.

Radioassay: Radioactive iron incorporation into the red cells was measured by injecting $^{59}FeCl_3$ in 1% sodium citrate: 0.2 uCi per mouse was injected intravenously, and a sample of blood was obtained by cardiac puncture 24-48 hours later. The activity in the blood samples was measured by a Packard Tricarb Gamma Spectrometer. The iron incorporation per mouse (as percent of the injected dose) was calculated according to the following formula:

$$\%^{59}Fe\ \text{Incorporation} = \frac{\text{Activity sample}}{\text{Activity standard}} \times \frac{\text{Wt animal}}{100} \times \frac{6.6 \times 8}{100}$$

A blood volume of 6.6 ml/100 gm body weight was assumed. (The activity of the standard represented 8% of the injected dose).

Irradiation Factors: 140 kV X-rays were used with the following factors: 5 mA., 0.1 mm Cu filter, 18 cm FSD. Dose rate 100 R/min; Mice were irradiated three at a time in a small plastic restrainer. Recipients of bone marrow received a dose of 650-710 R of whole body irradiation.

Bone Marrow suspension: The bone marrow suspension was prepared from the tibiae of donor mice by washing out with 0.5 ml of ice-cold Ringer's solution. The bone marrow was kept at 4-5° C throughout the whole procedure and was injected within one hour of obtaining it. Cells were dispersed in a homogeniser with a loosely fitting Teflon plunger, and then made up to the required concentration with cold Ringer's solution. All counts were performed before and after dilution using an improved Neubauer white blood cell counting chamber.

Production of anaemia in mice: Mice were bled by cardiac puncture, using light ether anaesthesia, 0.5 ml of blood being removed each time (i.e. about 1/3-1/2 of the blood volume). Immediate mortality from this procedure was about 10%, but animals that survived after the first hour survived indefinitely.

Production of plethora: Blood obtained by cardiac puncture was collected in heparin (100units/ml) and washed with physiological saline. Red blood cells were then resuspended in physiological saline to produce a volume equal to the original volume of blood. 0.5-1 ml of this suspension was injected i.v. prior to further procedures.

Results

Normal bone marrow cells (0, 2, 4, or 6×10^6 cells) were injected i.v. into recipient animals that had been given 650 R of whole body irradiation 24 hours earlier. Radioiron was injected i.v. after 5 days, and samples of blood were taken 48 hours later for radioassay. It can be seen from the results shown in table 1 *(fig. 1)* that iron uptake was linearly related to the dose of bone marrow cells injected, and that 6×10^6 bone marrow cells restored the iron uptake to the non-irradiated level (30-40%).

TABLE I

^{59}Fe Incorporation in whole body irradiated mice given various doses of normal bone marrow cells

Group	*N° cells injected*	*$\%^{59}Fe$ Incorporation**
I	2.1×10^6	13.6 ± 3.5
II	4.2×10^6	24.0 ± 1.6
III	6.2×10^6	29.4 ± 2.4
IV	—	4.4 ± 2.6
V	normal animals	30.5 ± 3.6

* Mean ± standard error of the mean.

This system lends itself admirably to to the study of bone marrow kinetics, since it is readily reproducible, and relatively simple to interpret. Examples of applications of this system include the rate of repopulation by a given dose of bone marrow cells in irradiated or otherwise treated recipient hosts, the effects of various factors such as anaemia ,anoxia, polycythaemia, or serum from such animals (or patients) on the repopulating ability of the transferred bone marrow; the radiosensitivity of bone marrow cells, etc. An example will be given here, illustrating the effect of anaemia on the repopulating potential of the bone marrow.

Mice were made anaemic by the removal of 1/3 of their blood volume by cardiac puncture, 5 hours, and 1, 2, 4, 6 and 8 days prior to the injection of radioactive iron. Twenty-four hours later, samples of blood were taken for radioassay. From table 2a it can be seen that there was a very pronounced variation in radioiron incorporation depending on the interval between the induction of the anaemia and the injection of the radioiron. Maximal stimulation occurred when the anaemia was induced 4 days before ^{59}Fe injection. Four million bone marrow cells from these mice were then transplanted into irradiated mice, and radioiron incorporation was measured 5 days later. Table 2b shows that there was no significant difference between the bone marrow from anaemic mice and those from normal donors ($P > 0.3$). This indicated that anaemia had no significant effect on the repopulating ability of bone marrow stem cells *(Fig. 2)*.

TABLE 2a

Effect of anaemia of variable duration on ^{59}Fe incorporation

Group	*Interval Anaemia ^{59}Fe injection*	% *^{59}Fe Incorporation*
I	5 hours	31.1 ± 1.5
II	1 day	21.9 ± 1.9
III	2 days	38.5 ± 4.5
IV	4 days	58.0 ± 6.0
V	6 days	40.8 ± 4.4
VI	8 days	28.6 ± 4.9
VII	normal mice	23.6 ± 0.8

TABLE 2b

Effects of bone marrow cells from above on ^{59}Fe incorporation in irradiated recipient mice

Group	*Interval Anaemia-Bone Marrow transfer*	% *^{59}Fe Incorporation*
I	1 day	29.6 ± 2.0
II	2 days	28.5 ± 2.1
III	3 days	27.6 ± 3.0
IV	5 days	20.4 ± 0.6
V	7 days	27.8 ± 2.9
VI	9 days	32.6 ± 2.4
VII	normal donors	24.0 ± 1.6
VIII	no bone marrow	4.4 ± 2.6

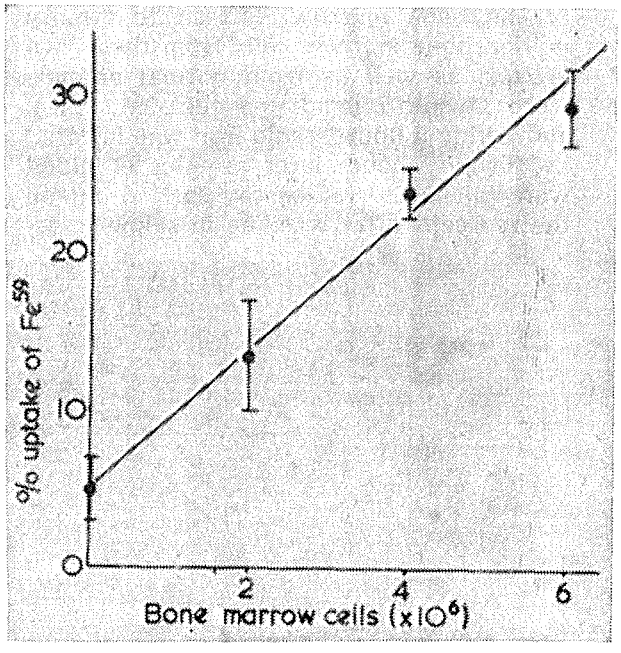

Fig. 1: Per cent ^{59}Fe incorporation in irradiated mice given various doses of normal bone marrow cells.

It is not to be concluded from the above that anaemia etc. has no effect on the bone marrow cellularity. The number of stem cells that are responsible for its repopulating ability make up a very small proportion of the total bone marrow — differentiated cells form by far the greatest proportion of bone marrow cells, and it is well known that anoxia and anaemia produce an intense stimulation of the differentiated bone marrow compartment. This is well illustrated in the following expe-

riment, where radioiron was injected soon after the transfer of bone marrow cells. This technique gives a measure of the differentiated (as opposed to stem cell), compartment, since the interval between bone marrow and iron injection was chosen to be short enough not to allow stem cells to divide and differentiate and appear in the peripheral blood as labelled erythrocytes.

Mice were rendered polycythaemic by the injection of one blood volume of washed red cells given on each of two successive days, 6 and 5 days prior to the transfer of the bone marrow. A second group of mice was made anaemic by cardiac puncture four days before transfer of the bone marrow. Fifteen to twenty million bone marrow cells from these two groups, as well as from normal animals were given to irradiated recipient mice, and within 5 hours, radio iron was injected i.v. Twenty hours later samples of blood were taken and radioassay carried out on the red cells after washing in saline to remove the activity present in the plasma.

From table 3 it can be seen that the differentiated compartment was much expanded in the bone marrow obtained from anaemic mice, since the uptake of radioiron was twice as high as in the normal group. There was no reduction in the ^{59}Fe incorporation in the polycythaemic group.

Discussion

The experiments described in this paper establish that radioiron incorporation into red cells is a linear function of the injected dose of the bone marrow cells. It was also seen that 5 days after the transplantation of 6×10^6 bone marrow cells, the erythroid activity in the irradiated recipients was equivalent to normal unirradiated controls, i.e., complete recovery can be assumed to have taken place .Since the generation time of these cells has been assessed to be about 16 hours (Cauchi, 1967) the total number of cells after 5 days would be expected to

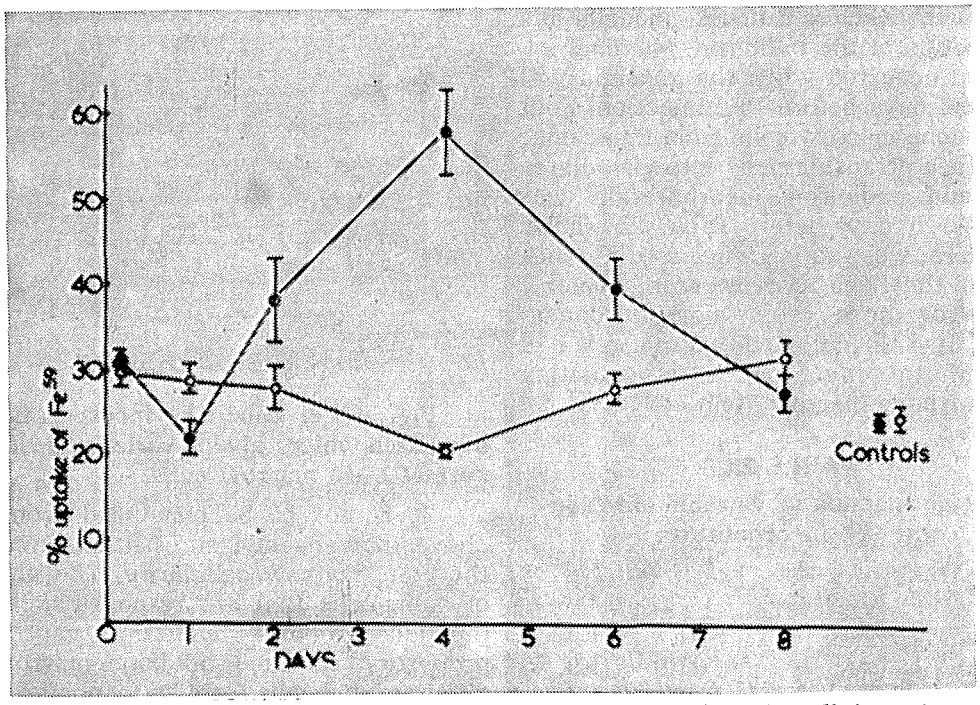

Figure 2: The upper graph (— ● —) shows the uptake of radioiron in anaemic mice. The interval between the induction of anaemia and the injection of ^{59}Fe is shown in the abscissa. The lower graph (—o—) represents the repopulating ability of bone marrow cells in irradiated mice given bone marrow cells from the previous groups of animals.

be about 768 × 10^6. By entirely different methods, Vogel (1961) has calculated that the total number of cells in the bone marrow of normal mice was about 36.0 × 10^9/kg body weight — for a 2 gm mouse, this is equivalent to 720 × 10^6 cells. Similarly, Pegg calculated a value of 39.8 × 10^9 cells/kg body weight — or 796 × 10^6 cells for a 20 gm mouse. Both these values, obtained by anatomical methods, are in a remarkable agreement with the above findings.

The interval between erythropoietic stimulation and maximal response varies with the nature of the stimulus employed. In these experiments, anaemia produced maximum stimulation after 4 days, whereas injection of exogenous erythropoietin (Hodgson and Eskuche, 1962) or sera from anaemic patients (Field *et al.*, 1968) produced maximal stimulation after 24 hours.

This time difference could be explained if anaemia did not stimulate erythroid precursors directly, but triggered off the production of erythropoietin by e.g. the kidneys: this endogenous erythropoietin then stimulated erythropoiesis.

TABLE 3

Effect of anaemia and polycythaemia on the differentiated compartment of the bone marrow

Group	*Type of Bone Marrow*	% ^{59}Fe *incorporation*	% *of Normal**
I	polycythaemic	1.6 ± 0.3	116
II	anaemic	4.6 ± 0.8	222
III	normal	2.1 ± 0.2	100
IV	no bone marrow	0.1 ± 0.01	—

* ^{59}Fe incorporation per 10^6 bone marrow cells transplanted, expressed as a percentage of normal (group III).

The greatly increased uptake of radioiron by differentiated bone marrow cells agrees with the well established morphological criteria. Bone marrow in anaemic subject is very cellular, being populated by differentiated cells of the erythroid series. It is not surprising therefore that ^{59}Fe incorporation was twice the normal value (table 3). It is interesting to note, however, that although the induction of polycythaemia by hypertransfusion leads to a reduction of the erythropoieic elements in the marrow (Alpen *et al.*, 1962), the cells from such bone marrow did not behave significantly different from normal bone marrow, indicating that there are as many differentiated cells in the polycythaemic, as in the normal bone marrow.

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